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## Sec-Mediated Secretion of Bacteriocin Enterocin P by *Lactococcus lactis*

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**Most lactic acid bacterium bacteriocins utilize specific leader peptides and dedicated machineries for secretion. In contrast, the enterococcal bacteriocin enterocin P (EntP) contains a typical signal peptide that directs its secretion when heterologously expressed in *Lactococcus lactis*. Signal peptide mutations and the SecA inhibitor azide blocked secretion. These observations demonstrate that EntP is secreted by the Sec translocase.**

Ribosomally synthesized antimicrobial peptides (bacteriocins) produced by lactic acid bacteria have potential applications as biopreservatives in the food industry (32). Enterocin P (EntP) is a class IIa bacteriocin (10) produced by *Enterococcus faecium* P13. It inhibits the growth of some food spoilage and food-borne pathogens, such as *Listeria monocytogenes*, *Staphylococcus aureus*, and *Clostridium botulinum* (5). The genetic determinants responsible for EntP production are organized in one operon comprising the bacteriocin structural gene, *entP*, and *orf2*, encoding a putative immunity protein (5). The EntP precursor (preEntP) consists of 71 amino acid residues (7.5 kDa) and contains a signal peptide of 27 amino acids that is removed proteolytically upon secretion, yielding the mature EntP (4.6 kDa).

The absence or presence (and type) of an N-terminal extension determines the secretion mechanism of class II bacteriocins. So far, the enterocins L50A, L50B, and Q (6) and the lactococcal bacteriocin LsbB (12) are the only examples of class II bacteriocins that are synthesized without N-terminal extension. The secretion mechanism of these enterocins is unknown, although LsbB has been reported to be secreted by LmrB, a multidrug resistance-like ABC transporter (12). Most class II bacteriocin precursors contain a double-glycine-type signal peptide and are translocated and processed by dedicated ABC transporters (15). Finally, preEntP and some other class II bacteriocins (5, 9, 22, 26, 37, 39) contain a typical Sec signal peptide (19) that is believed to direct these bacteriocins to the Sec translocase embedded in the cytoplasmic membranes (7). However, preEntP is a small, amphiphatic polypeptide that to some extent resembles procoat M13, the precursor of a small phage protein which does not utilize the Sec translocase despite containing a typical Sec-dependent signal sequence (2). Therefore, the objective of this work was to determine the secretion mechanism of preEntP.

### MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** The bacterial strains and plasmids used in this study are shown in Table 1. *E. faecium* P13 (5) and T136 (3)

were used as an EntP producer strain and sensitive indicator strain, respectively. Enterococci were grown at 30°C in MRS broth (Oxoid Ltd., Basingstoke, United Kingdom). *Lactococcus lactis* NZ9000 was used in combination with the nisin-controlled expression system (20) for protein overexpression. *L. lactis* was grown at 30°C in M17 medium (Difco, Le Pont de Claix, France) supplemented with 0.5% (wt/vol) glucose and 5 µg of chloramphenicol per ml when appropriate. Chemically defined medium (CDM) (21) was used to grow *L. lactis* for pulse-labeling experiments.

**Recombinant DNA techniques.** Cloning and DNA manipulation were performed as described by Sambrook et al. (36). Oligonucleotide primers used for PCR are shown in Table 2. *E. faecium* P13 and *L. lactis* MG1363 (13) chromosomal DNAs were used as templates for the PCR amplification of *entP* (5) and *usp45* (38), respectively. The PCR overlap extension method (17) was used to introduce single-amino-acid substitutions and to construct gene fusions of the signal peptides of preEntP (SP<sub>Ent</sub>) and preUsp45 (SP<sub>Usp</sub>) to the mature regions of preUsp45 and preEntP, respectively. PCR products were cloned in frame with a C-terminal hexahistidine tag on the pHLPS expression plasmid (33). Sequencing of the PCR-amplified DNA fragments was performed at Westburg Genomics (Leusden, The Netherlands).

**Purification of histidine-tagged EntP and Usp45.** Ammonium sulfate (45% [wt/vol]) was gradually added to the culture supernatants of *L. lactis* NZ9000 cells producing C-terminal hexahistidine-tagged EntP (EntP-His) or Usp45 (Usp45-His). After centrifugation, the precipitated proteins were resuspended in 50 mM sodium phosphate buffer (pH 8.0) with 300 mM NaCl (buffer A). Fractions were desalted by gel filtration (G-25 PD-10 columns, Amersham Biosciences, Uppsala, Sweden) and mixed with Ni-nitrilotriacetic acid (NTA) resin (QIAGEN, Hilden, Germany) equilibrated with buffer A (pH 8.0), and suspensions were gently shaken overnight at 4°C. The resin was transferred to a Bio-spin column (Bio-Rad, Hercules, Calif.) and subsequently washed with 10 column volumes of buffer A (pH 8.0) containing 75 or 10 mM imidazole for EntP-His and Usp45-His, respectively. Proteins were eluted with buffer A (pH 7.0) containing either 1 or 0.25 M imidazole for EntP-His and Usp45-His, respectively. Eluted fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting using monoclonal antibodies directed against the hexahistidine tag (Dianova, Hamburg, Germany) for chemiluminescence detection with the Lumi-Imager F1 (Roche Diagnostics). N-terminal sequencing of EntP was performed at the University of British Columbia (Vancouver, Canada).

**Pulse-labeling analysis.** Overnight cultures of *L. lactis* cells transformed with the appropriate plasmids and grown on CDM were freshly diluted (1:40) in 5 ml of the same medium. After incubation at 30°C to an optical density at 660 nm of 0.6, cells were collected by centrifugation, washed, and resuspended in CDM without methionine. Nisin was added, and incubation at 30°C was continued for 30 min. Where stated, cells were treated with sodium azide prior to the pulse to inhibit Sec-dependent protein secretion. Pulse-labeling was performed by the addition of 16 µCi of the Pro-mix L-(<sup>35</sup>S) in vitro cell labeling mix per ml (Amersham Biosciences, Roosendaal, The Netherlands). During the chase, non-radioactive methionine (25 mM) and cysteine (10 mM) were added, and at various times, 500-µl samples were removed and immediately centrifuged. The proteins present in the supernatants were precipitated with trichloroacetic acid (TCA; 10% [vol/vol]). Precipitates were washed with 80% (vol/vol) ice-cold acetone, dried, resuspended in SDS-PAGE sample buffer, and analyzed by SDS-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference
<b>Strains</b>		
<i>E. faecium</i>		
P13	Enterocin P producer	5
T136	Enterocin P sensitive	3
<i>L. lactis</i>		
MG1363	Plasmid-free derivative of SH4109	13
NZ9000	MG1363 derivative; <i>nisRK</i>	20
<b>Plasmids</b>		
pHLP5	Expression vector with histidine-tagged <i>lmrP</i> under control of $P_{nisA}$	33
pEK407	pHLP5 derivative; <i>entP</i>	This work
pEK410	pHLP5 derivative; <i>usp45</i>	This work
pEK411	pHLP5 derivative; fusion between SP <sub>Ent</sub> and mature Usp45	This work
pEK412	pHLP5 derivative; fusion between SP <sub>Usp</sub> and mature EntP	This work
pEK413	pHLP5 derivative; I11N mutant of <i>entP</i>	This work
pEK414	pHLP5 derivative; G12K mutant of <i>entP</i>	This work
pEK415	pHLP5 derivative; I13N mutant of <i>entP</i>	This work

PAGE and phosphorimaging. Cell pellets were resuspended in 100  $\mu$ l of lysis buffer (50 mM NaCl, 10 mM EDTA, 20% [wt/vol] sucrose, 10 mM Tris, pH 8.1, and 10 mg of lysozyme per ml) and incubated at 50°C for 10 min. Next, 10  $\mu$ l of 10% (wt/vol) SDS was added, and after boiling for 10 min, the samples were diluted 10 times with buffer A (pH 8.0) containing 0.05% (wt/vol) dodecyl- $\beta$ -D-maltoside (Anatrace, Maumee, Ohio) and 100  $\mu$ l of pre-equilibrated Ni-NTA resin. After 2 h at 4°C with shaking, proteins were eluted with buffer A (pH 7.0) containing 0.05% (wt/vol) DDM and 250 mM imidazole and further analyzed as described above.

**Antimicrobial activity assays.** The antimicrobial activity of EntP-His was measured by the agar well-diffusion assay (4) and quantified by a microtiter plate assay (18) using *E. faecium* T136 (3) as the indicator microorganism.

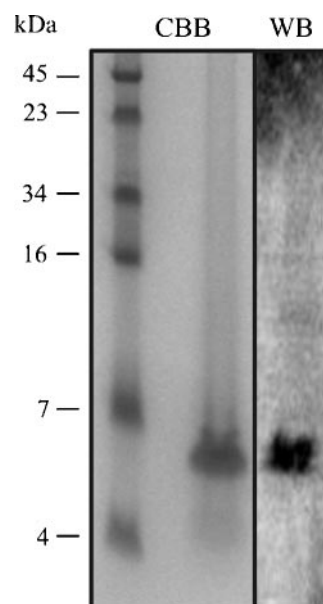


FIG. 1. Production of active EntP-His in *L. lactis*. Coomassie brilliant blue-stained Tricine-SDS-PAGE (CBB) and Western blotting (WB) show EntP-His purified from the supernatant of nisin-induced *L. lactis* NZ9000 expressing *entP*.

## RESULTS

**Heterologous expression of *entP* in *L. lactis*.** Production of EntP-His (6 kDa) in *L. lactis* NZ9000 was achieved when cells containing *entP* were induced with nisin. Coomassie-stained SDS-PAGE of the protein purified by Ni-NTA affinity chromatography showed a single band that corresponded to EntP-His (Fig. 1), as verified by N-terminal sequence analysis of the first six amino acid residues. This result indicates that preEntP is processed by *L. lactis* in an identical manner as in *E. faecium*. The heterologously produced EntP-His displayed antimicrobial activity against *E. faecium* T136 (not shown).

**Signal peptide swapping between preEntP and preUsp45.** Hybrid genes encoding the precursor proteins composed of the SP<sub>Ent</sub> fused to the mature Usp45 (*entP-usp45*) and vice versa

TABLE 2. Primers used in this study<sup>a</sup>

Primer	Sequence
ENTP-FW.....	5'-GGACCATGGAAGAAAAAATTATTTAGTTTACG-3'
ENTP-RV.....	5'-CACGGATCCATGTCCCATACCTGCCAAACC-3'
U45-FW.....	5'-GGAACCATGGGAAAAAAGATTATCTCAG-3'
U45-RV.....	5'-CGCGGGATCCGTTTGGCATCAAGAATGTTAC-3'
SPU45-RV.....	5'-CATATGAACGCGTAGCAGCGTAGACCCCTGAC-3'
MAU45-FW.....	5'-CAAAAGTTGATGCAGACACAAATTCAGATA-3'
SPENTP-RV.....	5'-TATCTGAATTTGTGTCTGCATCAACTTTTGTA-3'
MAENTP-FW.....	5'-GTCAGGGGTCTACGCTGCTACGCGTTCATATG-3'
Asn 11-FW.....	5'-GTTTAGCTCTTAATGGAATATTTGGGTTAG-3'
Asn 11-RV.....	5'-CTAACCCAAATATTCCATTAAGAGCTAAAC-3'
Lys 12-FW.....	5'-GTTTAGCTCTTATTAAGATATTTGGGTTAG-3'
Lys 12-RV.....	5'-CTAACCCAAAATCTTAATAAGAGCTAAAC-3'
Asn 13-FW.....	5'-GTTTAGCTCTTATTGGAAATTTGGGTTAGTTG-3'
Asn 13-RV.....	5'-CAACTAACCCAAAATTTCCAATAAGAGCTAAAC-3'

<sup>a</sup> Restriction sites for NcoI (forward primers; FW) and BamHI (reverse primers; RV) are underlined. Mismatched nucleotides introducing single-amino-acid substitutions in the SP<sub>Ent</sub> are shown in boldface.

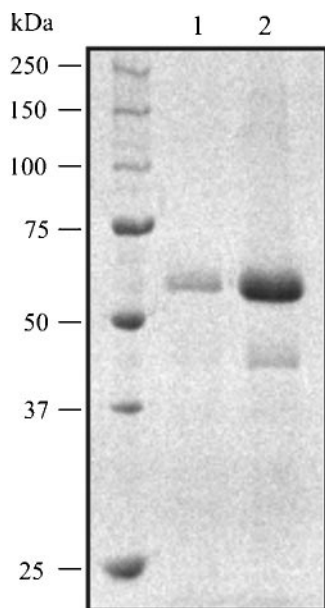


FIG. 2. Secretion of Usp45-His directed by the SP<sub>Ent</sub> or the SP<sub>Usp</sub>. Coomassie brilliant blue-stained SDS-PAGE (12% polyacrylamide) showing Usp45-His purified from the supernatant of *L. lactis* NZ9000 expressing SP<sub>Ent</sub>-Usp45-His (lane 1) or preUsp45-His (lane 2).

(*usp45-entP*), were constructed. Expression of *entP-usp45* resulted in high-level production of a protein of 50 to 75 kDa (Fig. 2) that corresponded to Usp45-His, as verified by Western blotting (results not shown). Although the native signal peptide supported more efficient secretion of Usp45-His (Fig. 2), the data show that the SP<sub>Ent</sub> can direct the secretion of a Sec-dependent protein. Nisin induction of *L. lactis* cells containing *usp45-entP* resulted in the production of EntP-His (Fig. 3A), which displayed a normal antimicrobial activity (Fig. 3B). These results show that the SP<sub>Ent</sub> and the SP<sub>Usp</sub> are functionally exchangeable and indicate that the SP<sub>Ent</sub> is a typical Sec-dependent signal peptide.

**Secretion of EntP-His directed by mutated signal peptides.** To further investigate the characteristics of the SP<sub>Ent</sub>, point mutations were introduced into its hydrophobic core. Replacement of the isoleucine residues at positions 11 and 13 with asparagine and of the glycine at position 12 with lysine, yielded the mutated signal peptides I11N, I13N, and G12K, respectively. Analysis of the secretion of EntP-His by a microtiter plate assay showed that only 0.6, 0.5, and 2.5% of the antimicrobial activity produced by the cells expressing the wild-type precursor was obtained when secretion was directed by the mutated signal peptides I11N, G12K, and I13N, respectively.

**Effect of azide on EntP secretion.** Azide, a potent inhibitor of SecA, blocks Sec-dependent protein secretion in *Escherichia coli* and *Bacillus subtilis* (29, 31). The effect of azide on the SP<sub>Ent</sub>-mediated secretion in *L. lactis* was evaluated by using pulse-labeling assays. Secretion of EntP-His or Usp45-His directed by the SP<sub>Ent</sub> was drastically reduced in the presence of azide (Fig. 4A and B), while for SP<sub>Ent</sub>-Usp45-His, the amount of precursor remaining inside the cells increased (Fig. 4C). Similarly, secretion of preUsp45-His was blocked by azide (re-

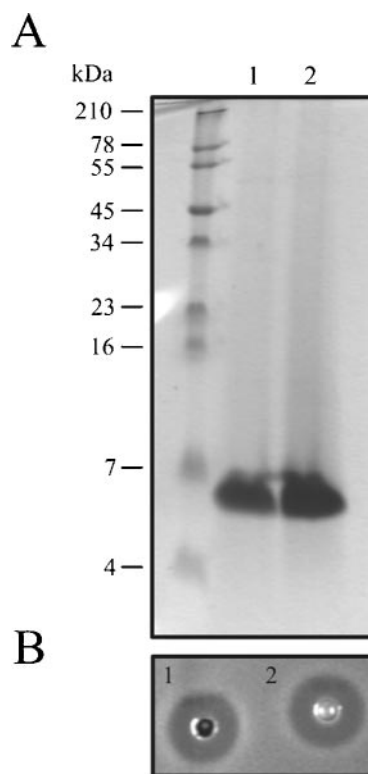


FIG. 3. Secretion of EntP-His directed by the SP<sub>Ent</sub> or the SP<sub>Usp</sub>. (A) Coomassie-brilliant blue-stained Tricine-SDS-PAGE showing EntP-His purified from the supernatant of *L. lactis* NZ9000 expressing preEntP-His (lane 1) or SP<sub>Usp</sub>-EntP-His (lane 2). (B) Agar diffusion test comparing the antimicrobial activity of the proteins shown in panel A against *E. faecium* T136.

sults not shown). These data indicate that EntP secretion in *L. lactis* is mediated by the Sec translocase.

## DISCUSSION

In this report, we have investigated the mechanism of secretion of the heterologously produced EntP-His in *L. lactis*. EntP belongs to the class II bacteriocins that are synthesized as precursors with an N-terminal extension that shows the typical tripartite structure of Sec-dependent signal peptides (19) (Table 3). The assumption of a Sec-dependent secretion of bacteriocin 31 and acidocin B is supported by their production by heterologous hosts in which only the bacteriocin structural and immunity genes have been introduced (22, 37). For divergicin A, additional evidence is provided by the ability of its signal peptide to direct the secretion of Sec-dependent (39) and Sec-independent (1, 27, 28) proteins. In the case of EntP, the presence of a seemingly typical signal sequence (5) and the lack of dedicated transport genes in the *entP* operon (16) suggested that this bacteriocin is secreted via the Sec pathway. However, there are some unusual characteristics in the preEntP amino acid sequence which prompted us to investigate its secretion mechanism. First, there is a positively charged residue at the +3 position of the mature EntP. Although this feature is not uncommon in class II bacteriocins secreted by dedicated ABC transporters, the presence of pos-

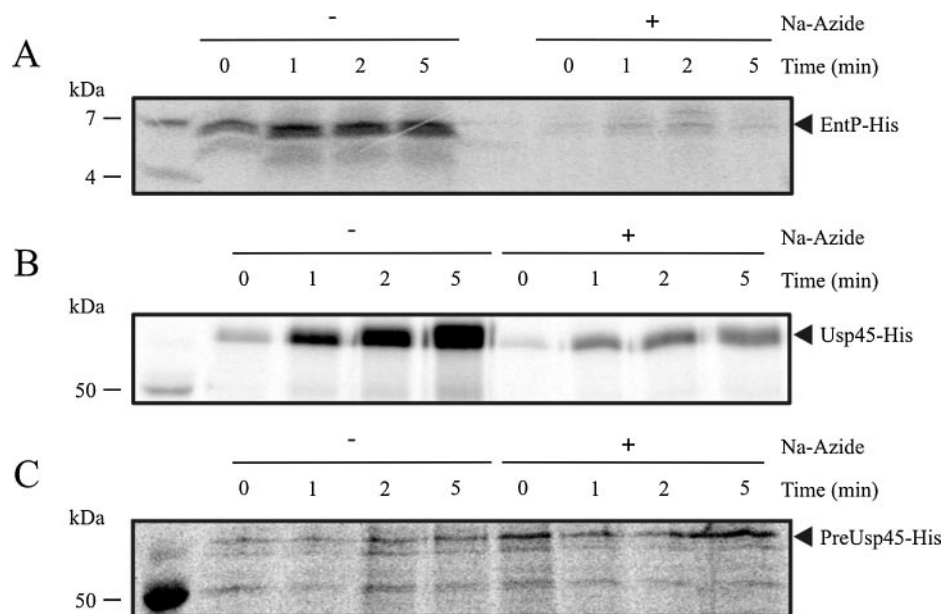


FIG. 4. Effect of sodium azide on the SP<sub>Ent</sub>-mediated secretion. *L. lactis* NZ9000 cells expressing SP<sub>Ent</sub>-EntP-His (A) or SP<sub>Ent</sub>-Usp45-His (B and C) were pulse-labeled with a mixture of radioactive methionine and cysteine. Where indicated, cells were treated with 3 mM sodium azide 1 min prior to the pulse. Samples were taken at the indicated times (in minutes) after the chase, and the supernatants (A and B) and the cell pellets (C) were processed as described in the text.

itively charged amino acids in the N-terminal region of the mature proteins negatively affects Sec-dependent secretion in *E. coli* (25) and *L. lactis* (23, 24). Second, a lysine residue is present at position -4 relative to the cleavage site in preEntP. This attribute is not observed in other putative Sec-dependent class II bacteriocins and would render the signal peptide inactive for Sec-dependent secretion in *E. coli*. Finally, asparagine, a residue rarely present in prokaryotic signal peptides (14), is located in the hydrophobic core of SP<sub>Ent</sub>.

Since no dedicated transporter has been defined for EntP, the signal sequence should support its secretion via the Sec translocase when expressed in a heterologous host. Indeed, *L. lactis* could secrete EntP-His either with the native or with the Usp45 signal peptide. On the other hand, secretion of a typical Sec substrate as Usp45 (38) is supported by the SP<sub>Ent</sub>. Finally, secretion is dramatically reduced by signal sequence mutations that interfere with the secretion of Sec-dependent substrates in *E. coli* (14) and is blocked by the SecA inhibitor azide. Taken together, these results lend strong support for the hypothesis that EntP is secreted into the medium by the Sec translocase.

In an attempt to detect special features of the SP<sub>Ent</sub> that

could be required for the bacteriocin secretion, the ability of the SP<sub>Usp</sub> to direct EntP-His secretion was evaluated. Previous work using SP<sub>Usp</sub> for heterologous protein expression showed that the combination of this signal peptide with a negatively charged N terminus in the mature protein increased the secretion efficiency (8, 24, 34). Combination with the cationic N terminus of EntP appears at least as efficient as the native SP<sub>Ent</sub>. Apparently, the positive charge in the mature amino terminus does not interfere with a proper functioning of the SP<sub>Usp</sub> in *L. lactis*.

Several bacteriocins have been heterologously produced in *L. lactis*, using an ABC-dedicated transport system (reviewed in reference 35). However, a limited number (28, 30, 39) have been produced through secretion by the Sec system. This study shows that *L. lactis* is an excellent host for the production of Sec-dependent bacteriocins. Since *L. lactis* is not sensitive to EntP (5), the bacteriocin could be expressed without the immunity protein Orf2. This makes the food-grade microorganism *L. lactis* an ideal host for the heterologous production of EntP, thereby avoiding concerns about the safety of enterococci in food (11).

TABLE 3. Signal sequences of putative class II Sec-dependent bacteriocins

Bacteriocin	Amino acid sequence <sup>a</sup>	Reference
Acidocin B	MVTKYGRNLGLSKVELFAIWAVLVVALLATA ↓ NIYWI	22
Divergicin A	MKKQILKGLVIVVCLSGATFFSTPQQASA AAPKI	39
Bacteriocin 31	MKKKLVICGIIGIGFTALGTNVEA ATYYG	37
Enterocin P	MRKKLFSALIGIFGLVVTNFGTKVDA ATRSY	5
Lactococcin 972	MKTKSLVLALSAVTLFSAGGIVAQA EGTWQ	26
Enterocin SE-K4	MKKKLKGLVICGMIGIGFTALGTNVEA ATYYG	9

<sup>a</sup> Shown are the amino acid sequence of the signal peptides and the first five residues after the processing site, which is indicated by an arrow.



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